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The study of microbiologically influenced corrosion (MIC) has progressed from phenomenological case histories to a mature interdisciplinary science including electrochemical, metallurgical, surface analytical, microbiological, biotechnological, and biophysical techniques. With microelectrodes and gene probes it is now possible to measure interfacial dissolved oxygen, dissolved sulfide and pH, and to determine microbial species responsible for localized chemistry. Biofilms can be tailored to contain consortia of specific microorganisms and naturally-occurring biofilms can be dissected into cellular and extracellular constitutents. Scanning vibrating electrodes can be used to map the distribution of anodic electrochemical activity. Electrochemical impedance spectroscopy and electrochemical noise analysis techniques have been developed to non-destructively evaluate localized corrosion due to MIC. The development of environmental scanning electron, atomic force, and laser confocal microscopy makes it possible to image cells on surfaces and to accurately determine the spatial relationship between microorganisms and localized phenomena. Transport of nutrients through biofilms can be modeled using techniques including optical density measurements to precisely locate the water/biofilm interface and nuclear magnetic resonance imaging to visualize flow characteristics near surfaces colonized with microorganisms. The ways in which new techniques can be used to understand fundamental mechanisms and to discriminate MIC will be discussed in this paper.

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Keynote Address

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Advances in MIC Testing

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ABSTRACT: The study of microbiologically influenced corrosion (MIC) has progressed from phenomenological case histories to a mature interdisciplinary science including electrochemical, metallurgical, surface analytical, microbiological, biotechnological, and biophysical techniques. With microelectrodes and gene probes it is now possible to measure interfacial dissolved oxygen, dissolved sulfide and pH, and to determine microbial species responsible for localized chemistry. Biofilms can be tailored to contain consortia of specific microorganisms and naturally-occurring biofilms can be dissected into cellular and extracellular constituents. Scanning vibrating electrodes can be used to map the distribution of anodic electrochemical activity. Electrochemical impedance spectroscopy and electrochemical noise analysis techniques have been developed to non-destructively evaluate localized corrosion due to MIC. The development of environmental scanning electron, atomic force, and laser confocal microscopy makes it possible to image cells on surfaces and to accurately determine the spatial relationship between microorganisms and localized phenomena. Transport of nutrients through biofilms can be modeled using techniques including optical density measurements to precisely locate the water/biofilm interface and nuclear magnetic resonance imaging to visualize flow characteristics near surfaces colonized with microorganisms. The ways in which new techniques can be used to understand fundamental mechanisms and to discriminate MIC will be discussed in this paper.

KEYWORDS: microbiologically influenced corrosion (MIC), culture techniques, electrochemistry, surface analyses

Introduction

Corrosion associated with microorganisms has been recognized for over 50 years, yet the study of MIC is a relatively new, multidisciplinary field. In 1985 an International Conference on Biologically Influenced Corrosion was sponsored by the National Association of Corrosion Engineers (NACE). Of the thirty-six papers in the proceedings volume, roughly half the papers are descriptive case studies and ten titles contain the words sulfate-reducing bacteria (SRB) [*1*]. In 1985 many of the techniques for evaluating MIC depended on characterizing shape, color, and smell of surface deposits in addition to the presence of specific types of bacteria, usually SRB. In contrast, today there is general recognition that SRB contribute and control many cases of MIC, but that it is the total community of bacteria within the biofilm that is responsible for MIC and there is no correlation between numbers and types of cells and localized corrosion [*2*]. Electrochemical, surface analytical, and microbiological techniques are now routinely combined to elucidate the complexities of microbial interactions with metal substrata.

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Techniques for Determining Cellular Constituents Within Biofilms

Culture Techniques

For many years, the standard for evaluating MIC has been the enumeration of SRB either in bulk liquids or in surface deposits using a liquid or solid [*3*] medium with sodium lactate as the carbon source [*4,5*]. When SRB are present in the sample, sulfate is reduced to sulfide which reacts with iron in solution to produce black ferrous sulfide. Blackening of the medium over a 28-day period signals the presence of SRB. Usually, 1 mL samples are injected by syringe into media bottles for 10-fold dilutions. It is assumed that only a single living bacterium is required to blacken a bottle. The simplest interpretation of test results is to consider that if one bottle is blackened, the sample contained at least 1 organism, if two bottles are blackened, the sample contained 10 organisms; three bottles, 100 organisms and so on. Agar slants can be inoculated by dipping a pipe cleaner into a liquid sample and inserting it into a single vial of solid or semi-solid agar. Mineral oil and a CO_2-generating tablet are usually added to exclude oxygen, and the vial is capped, incubated for 5 days, and checked daily for blackening.

The distinct advantage of culturing techniques is that they are extremely sensitive. Low numbers of SRB grow to easily detectable higher numbers in the proper culture medium. However, growth media tend to be strain-specific. For example, lactate-based media sustain the growth of lactate oxidizers but not acetate-oxidizing bacteria. Incubating at one temperature is further selective. Culturing methods using agar media cannot distinguish between a single SRB cell and a clump of SRB cells [*3*]. The present trend in culture techniques is to attempt to culture several physiological groups including aerobic, heterotrophic bacteria; facultative anaerobic bacteria, and acid-producing bacteria in addition to sulfate-reducing bacteria [*6*]. A complex SRB medium containing multiple carbon sources that can be degraded to both acetate and lactate has been developed and compared to five other commercially available media using natural and produced waters and surface deposits [*7*].

Biochemical Assays

Biochemical assays have been developed for the detection of specific microorganisms associated with MIC. Unlike culturing techniques, biochemical assays for detecting and quantifying bacteria do not require growth of the bacteria. Instead, biochemical assays measure constitutive properties including adenosine triphosphate (ATP) [*8*], phospholipid fatty acids (PLFA) [*2*], cell-bound antibodies [*9,10*], and DNA [*11*]. Adenosine-5'-phosphosulfate (APS) reductase [*3*], hydrogenase [*12*], and radiorespirometric measurements have been used to estimate SRB populations and activity [*13,14*].

ATP assays estimate the total number of viable organisms by measuring the amount of adenosine triphosphate in a sample. ATP is a compound found in all living matter. The procedure requires that a water sample be filtered to remove solids and salts which may interfere with the test. The filtered sample is added to a reagent that releases cell ATP. An enzyme then reacts with the ATP to produce a photochemical reaction. Emitted light is measured with a photometer and the number of bacterial cells is estimated from the total light emitted.

Biofilm community structure can be analyzed using cluster analysis of the PLFA profiles [*2*]. PLFA profiles for natural biofilms have been shown to be more complex than profiles for laboratory biofilms. None of the laboratory profiles clustered closely with profiles from natural biofilms. In addition, the PLFA profiles for attached bacteria clustered separately

from profiles of the same bacteria in the bulk phase, suggesting that either the community or the physiology of attached bacteria differ from that of bulk phase bacteria.

Immunofluorescence techniques have been developed for the identification of specific bacteria in biofilms [*15,16*]. Epifluorescence cell surface antibody (ECSA) methods for detecting SRB are based on the use and subsequent detection of specific antibodies, produced in rabbits, that react with SRB cells [*9,10*]. A secondary antibody, produced in goats, is then reacted with the primary rabbit antibodies bound to SRB cells. In some cases, goat antibodies are linked to a fluorochrome which enables bacterial cells marked with the secondary antibody to be viewed with an epifluorescence microscope. In other cases, goat antibodies are conjugated with an enzyme (alkaline phosphatase) that can then be reacted with a colorless substrate to produce a visible color proportional to the quantity of SRB present. The detection limits for the field test are 10 000 SRB/mm^2 filter area. The color reagent used for the field tests is unstable at room temperature and tends to bind nonspecifically with antibodies adsorbed directly at active sites on the filter, creating a false positive that may interfere with the detection of SRB at levels below 10 000 cells/mm^2. Antigenic structures of marine and terrestrial strains are distinctly different and therefore antibodies to either strain did not react with the other. Furthermore, SRB antibodies did not react with non-SRB bacteria. The developers report a poor response of rabbit antibodies developed from pure SRB cultures to mixed populations [*10*]. Rabbit SRB antibodies generated from fresh SRB strains from Prudhoe Bay, Alaska, as well as terrestrial and marine locations, were found to react better with SRB from natural sources. It is possible to differentiate individual species within a biofilm by reacting them with monoclonal antibodies specific to outer cell membrane antigens. Hogan [*11*] described a non-isotopic semi-quantitative procedure for the detection of *Desulfobacterium* and *Desulfotomaculum* using DNA probes labeled with an acridinium ester that is sensitive to 10^4 organisms per mL.

Direct molecular characterization of natural microbial populations can be accomplished with sequence analysis of 5S rRNAs [*17,18*]. More recently, fluorescent dye-labeled oligonucleotide probes have been used for microscopic identification of single cells and characterization of mixed populations. Polymerase chain reaction amplification, comparative sequencing and whole cell hybridization have been combined to selectively identify and visualize SRB both in established and developing multispecies biofilms [*19*].

APS reductase is an intercellular enzyme found in all SRB. Briefly, cells are washed to remove interfacing chemicals, including hydrogen sulfide, and lysed to release APS reductase. The lysed sample is washed, added to an antibody reagent and exposed to a color-developing solution. In the presence of APS reductase a blue color appears within 10 min. The degree of color is proportional to the amount of enzyme and roughly to the number of cells from which the enzyme was extracted. Similarly, a procedure has been developed to quantify hydrogenase from hydrogenase-positive SRB requiring cells to be concentrated by filtration from water samples [*12*]. Solids, including corrosion product and sludge, can be used without pretreatment. The sample is exposed to an enzyme extracting solution for 15 min and placed in an anaerobic chamber from which oxygen is removed by hydrogen. The enzyme reacts with excess hydrogen and simultaneously reduces an indicator dye in solution. The activity of the hydrogenase is established by the development of a blue color in less than 4 h. The intensity of the blue color is proportional to the rate of hydrogen uptake by the enzyme. The technique does not attempt to estimate specific numbers of SRB.

Roszak and Colwell [*20*] reviewed techniques commonly used to detect microbial activities in natural environments, including transformations of radiolabelled metabolic precursors. Phelps et al. [*21*] and Mittelman et al. [*22*] used uptake or transformation of ^{14}C-labelled metabolic precursors to examine activities of sessile bacteria in natural environments and

in laboratory models. Phelps et al. [*21*] used a variety of ^{14}C-labelled compounds to quantify catabolic and anabolic bacterial activities associated with corrosion tubercles in steel natural gas transmission pipelines. They demonstrated that organic acid was produced from H_2 and CO_2 in natural gas by acetogenic bacteria, and that acidification could lead to enhanced corrosion of the steel. Mittelman et al. [*22*] used measurement of lipid biosynthesis from ^{14}C-acetate, in conjunction with measurements of microbioal biomass and extracellular polymer, to study effects of differential fluid shear on physiology and metabolism of *Alteromonas* (formerly *Pseudomonas*) *atlantica*. Increasing shear force increased the rate of total lipid biosynthesis, but decreased per cell biosynthesis. Increasing fluid shear also increased cellular biomass and greatly increased the ratio of extracellular polymer to cellular protein.

Techniques for analyzing microbial metabolic activity at localized sites are also being developed. Franklin et al. [*23*] incubated microbial biofilms with ^{14}C-metabolic precursors and autoradiographed the biofilms to locate biosynthetic activity on corroding metal surfaces. The uptake of the labelled compounds was related to localized electrochemical activities associated with corrosion reactions.

A major breakthrough in determining bacterial activity within biofilms has been the use of "reporter" genes that can signal the induction of specific metabolic pathways. King et al. [*24*] engineered the incorporation of a promotorless cassette of *lux* genes into specific operons of *Pseudomonas* so that these operons induce bioluminescence during the degradation of naphthalene. Mittelman et al.[†] used the bioluminescent reporter gene to provide a quantitative measure of attachment of microorganisms onto metal and glass surfaces in a laminar flow system. They found that biofilm light production was directly correlated with biofilm cell numbers in a range of 10^5–10^7 cells/cm^2. Using reporter genes, Marshall et al. [*25*] demonstrated that bacteria immobilized at surfaces exhibit physiological properties not found in the same organisms in the aqueous phase. Some genes are turned on at a solid surface despite not being expressed in liquid or on solid media. It is also likely that other genes are turned off at surfaces. They identified acid- and alkali-inducible genes in *E. coli*. Marshall et al. [*25*] further demonstrated gene transfers within biofilms even in the absence of imposed selection pressure.

Rosser and Hamilton [*13*], with subsequent modifications [*14*], developed a test tube technique for a ^{35}S sulfate radiorespirometric assay to measure SRB metabolic activity on the surface of metal coupons after exposure to corrosive environments. The coupon is placed into anaerobic filtered sterile seawater containing ^{35}S-sulfate. Oxygen-free zinc acetate is immediately injected onto an enclosed filter paper wick and the entire system is incubated. Oxygen-free hydrochloric acid is then injected past the wick into the solution. Volatile acid sulfides, including any $H_2{}^{35}S$ formed, are trapped during an equilibration period. The wick is removed from the tube and the radioactivity measured using a liquid scintillation counter, after which the sulfate reduction rate is calculated. This technique has been used for both bulk and coupon samples.

Techniques for Identification and Measurement of MIC

Electrochemical Techniques

Mansfeld and Little [*26*] recently reviewed electrochemical techniques applied to MIC studies and no attempt will be made to discuss all the innovations in electrochemical techniques. Three nondestructive electrochemical techniques, the scanning vibrating electrode technique (SVET), electrochemical impedance spectroscopy (EIS), and electrochemical

[†] M. W. Mittelman, J. M. H. King, G. S. Savler, and D. C. White, unpublished data, University of Tennessee, Knoxville, TN, 1992.

noise analysis (ENA) are currently being used to provide unique insights into mechanisms for MIC.

SVET is used to determine the magnitude and sign of current densities over freely corroding metals in solution [27]. Franklin et al. [28] used SVET to show a spatial relationship between localized corrosion and bacterial cells on carbon steel surfaces. Pit propagation depended on the presence of bacteria. The authors proposed that biofilms inhibited migration of aggressive ions from pits or migration of inhibiting ions from the bulk solution into pits.

EIS techniques record impedance data as a function of the frequency of an applied signal at a fixed potential [29]. A large frequency range (65 kHz to 1 mHz) must be investigated to obtain a complete impedance spectrum. Dowling et al. [30] and Franklin et al. [31] demonstrated that the small signals required for EIS do not adversely affect the number, viability, and activity of microorganisms within a biofilm.

EIS data may be used to determine polarization resistance, the inverse of corrosion rate. Sophisticated models have been developed for localized corrosion [32,33] that provide additional information from EIS data. Several reports have been published in which EIS has been used to study the role of SRB in corrosion of buried pipes [34–36] and reinforced concrete [37–39]. The formation of biofilms and calcareous deposits on three stainless steels and titanium during exposure to natural seawater was followed using EIS and surface analysis [40,41]. Ferrante and Feron [42] used EIS data to conclude that the material composition of steels was more important for MIC resistance than bacterial population, incubation time, sulfide content, and other products of bacterial growth. Jones et al. [43] used EIS to determine the effects of several mixed microbiological communities on the protective properties of epoxy coatings on steel. A damage function was defined which allowed qualitative assessment of coating deterioration due to MIC.

ENA follows fluctuations of potential or current as a function of time or experimental conditions. Analysis of the structure of the electrochemical noise using the frequency dependence of the power spectral density can provide information concerning the nature of corrosion processes and magnitude of corrosion rate. King et al. [36] interpreted noise measurements for steel pipes in environments containing SRB as being indicative of film formation and breakdown. Iverson [44,45] used ENA to monitor corrosion of mild steel in a trypticase seawater culture of a marine SRB and concluded that breakdown of the iron sulfide film was accompanied by the generation of potential electrochemical noise.

Surface Analytical Techniques

Nivens et al. [46] demonstrated that attenuated total reflectance infrared spectroscopy (ATR-FT/IR) can be used to detect changes in sessile microbial biomass. The ATR-FT IR studies showed that changes in the physiological properties of attached bacteria were induced by changes in the bulk phase. They demonstrated that the number of attached *Caulobacter sp.* was directly correlated with the intensity of the infrared amide II asymmetrical stretch band at 1543 cm^{-1}, corresponding to bacterial protein. The technique was sensitive to 10^6 bacteria/cm^2, and changes in the physiological status of the attached bacteria could be measured. For example, production of the intracellular storage lipid, poly-B hydroxyalkanoate, and production of extracellular polymer, were monitored by absorbance at 1730 cm^{-1} (C=O stretch) and 1084 cm^{-1} (C—O stretch), respectively.

Geesey and Bremer [47] used ATR-FT/IR to evaluate non-destructively, in real time, interactions of bacteria with thin films of copper deposited on germanium. Changes in the thickness of the copper films were measured as increased intensity of the infrared water absorption band at 1640 cm^{-1}. The authors compared copper loss in the presence of bacteria

isolated from corroded copper samples and were able to observe differences between two cultures. Using this technique, Jolley et al. [*48*] observed copper oxidation by three polymers, including bacterial exopolymer.

Nivens et al. [*46*] investigated the use of the quartz crystal microbalance (QCM), a very sensitive mass-sensing device, for detecting attached microbial films. The QCM was more sensitive to changes in biomass than ATR-FT/IR, with a detection limit of 10^4 bacteria cm^{-2} and a linear range of at least two orders of magnitude. An interesting aspect of both ATR-FT IR and the QCM is that substrata of both techniques can be used for electrochemical analyses so that corrosion information can be obtained while changes in microbial biofilms are monitored.

It is now generally recognized that biofilms alter biofilm/metal interfacial chemistries. Direct chemical measurements are restricted by biofilm thickness and the heterogeneous anisotropic nature of biofilms [*49*]. Ion-selective and gas sensing microprobes with tip diameters less than 10 μm have been developed for direct biofilm measurements. Lewandowski [*49*] measured dissolved oxygen profiles in a continuous flow, open channel reactor with a mixed biofilm on a metal surface. Van Houdt et al. [*50*] developed a rugged iridium oxide pH microelectrode with a 3 to 5 μm tip diameter to measure a pH profile across a mixed population biofilm on a polycarbonate disc.

An in-situ microtechnique has been developed for evaluating parameters of diffusion-controlled reactions in biofilms [*51*]. A microprobe 15 μm in diameter was used to simultaneously measure dissolved oxygen and optical density at different depths in a submerged biofilm. The diffusion coefficient for dissolved oxygen, the dissolved oxygen flux, and the half velocity coefficient were then calculated.

Nuclear magnetic resonance imaging (NMRI), a non-invasive method, uses radiofrequency magnetic fields in the presence of a strong magnetic field to provide information about the concentration and physical state of specific atomic nuclei. Lewandowski et al. [*52*] demonstrated the use of NMRI to show distribution of water, flow velocities, and biomass in a biofilm/polycarbonate reactor system.

Recent developments in image analysis systems and electron, atomic and laser microscopies make it possible to image biological materials in the hydrated state. Muellar et al. [*53*] were able to determine rate coefficients for early bacterial colonization on copper, silicon, stainless steel and glass using a chemostat, a flow cell, and a microscope equipped with an image analysis system. Substrata were monitored using reflective light from a microscope equipped with a Nomarski lens and video camera recorder. Transmitted light was used for transparent surfaces. They demonstrated that surface roughness and surface free energy correlated positively with biological and abiological sorption processes.

Little et al. [*54*] used environmental scanning electron microscopy/energy-dispersive X-ray analysis (ESEM/EDS) to study biofilms on stainless steel surfaces, observing a gelatinous layer in which microalgae were embedded. Extracellular polymeric acidic polysaccharides bind and precipitate heavy metals. ESEM/EDS spectra indicated local concentrations of Al, Ni, and Ti. Images of the same specimens made using traditional scanning electron microscopy (SEM) demonstrated a loss of cellular and extracellular material. Dehydration of the biofilm with solvents, required for SEM, either extracted bound metals from the biofilm by ion exchange/solvent extraction or removed the metals with the extracellular polymeric material.

Laser confocal microscopy permits one to create three-dimensional images, see surface contour in minute detail, and accurately measure critical dimensions by mechanically scanning the object with laser light [55]. A sharply focused image of a single horizontal plane within a specimen is formed while light from out of focus areas is repressed from view. The process is repeated again and again at precise intervals on horizontal planes and the visual data from all images compiled to create a single, multidimensional view of the subject.

Geesey[1] used laser confocal microscopy to produce three-dimensional images of bacteria within scratches, milling lines and grain boundaries.

The atomic force microscope (AFM) is related to the scanning tunneling microscope (STM). The STM uses an atomically sharp conductive tip held angstroms from the surface to profile surface features with angstrom resolution. When the tip is electrically biased with respect to the sample, a current will flow between the surface atom closest to the tip and the nearest tip atom by the quantum mechanical process of electron tunneling. While the STM requires the sample to be electrically conductive or coated with a conductive material, the AFM can be used to image non-conducting surfaces and does not rely on tunneling current. AFM provides exceptional detail and allows viewing of specimens in the hydrated state. AFM uses an extremely sharp scanning probe mounted on a flexible cantilever to record x,y,z coordinates of a sample in fractions of a nanometer. Photodiode electrical outputs mimic sample topography and serve as the basis for the resulting image. AFM images of copper exposed to a bacterial culture medium for 7 days showed biofilms distributed heterogeneously across the surface with regard to both cell numbers and depth [*56*]. Bacterial cells were associated with pits on the surface of the copper coupons.

Conversion of metals to sulfides by SRB has been studied since the late 1800s [*57*]. Baas-Becking and Moore identified mackinawite, gregrite and smythite as indicators for SRB corrosion of ferrous metals in anaerobic environments [*58*]. McNeil et al. analyzed sulfide mineral deposits on copper alloys colonized by SRB in an attempt to identify specific mineralogies that were indicative of SRB activity [*59*]. They concluded that the formation of non-adherent layers of chalcocite (Cu$_2$S) and the presence of hexagonal chalcocite were indicators of SRB-induced corrosion of copper. The compounds were not observed abiotically and their presence in near-surface environments could not be explained thermodynamically.

Sulfur isotope fractionation was demonstrated by Little et al. in sulfide corrosion deposits resulting from the activities of SRB within biofilms on copper surfaces [*60*]. ^{32}S accumulated in sulfide-rich corrosion products, and ^{34}S was concentrated in the residual sulfate in the culture medium. Accumulation of the lighter isotope was related to surface derivatization or corrosion as measured by weight loss. Use of this and the preceeding mineralogical technique to identify SRB-related corrosion requires sophisticated laboratory procedures.

Conclusions

The combined testing approaches of microbiology, electrochemistry, and surface chemistry have been reviewed to provide insight into complex interactions between biofilms and metal surfaces. Multimedia microbiological cultures, biochemical assays and genetic probes are being used to demonstrate the presence of specific types of bacteria. ESEM, AFM and laser confocal microscopy have demonstrated the spatial relationship between bacteria and localized corrosion on hydrated surfaces. Dissolved oxygen, dissolved sulfides, pH and optical density profiles through biofilms have been made with microprobes. Electrochemical testing, including EIS, SVET and ENA, has been used to demonstrate MIC for many alloys in a large number of environments.

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DISCUSSION

Tom Jack[1] *(written discussion)*—I was interested by one subject that you did not mention but which is probably going to be very important: kinetics of transport phenomena in biofilms. Could you comment on the state of the art?

B. Little and P. Wagner (authors' closure)—Techniques for determining biofilm reaction kinetics and the related diffusion coefficients depend on two types of testing: (1) chemical analyses of bulk water and (2) measurements inside the biofilm using microsensors. Le-

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wandowski et al. developed a microtechnique that allows evaluation of diffusion-controlled reactions within biofilms. They presented an algorithm and instrumentation for measuring respiration reaction kinetics in biofilms and simultaneously measured dissolved oxygen and optical density through a biofilm. The biofilm diffusion coefficient for dissolved oxygen, the dissolved oxygen flux through the biofilm surface and the half velocity coefficient were calculated. The procedure is general and can be used for organic compounds or dissolved gases for which a concentration profile across a biofilm can be measured. See "Reaction Kinetics in Biofilms" by Z. Lewandowski, G. Walser and W. Characklis in *Biotechnology and Bioengineering*, Vol. 38, 1991, pp. 877-882.